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Multisite Clickable Modification of Proteins Using Lipoic Acid Ligase

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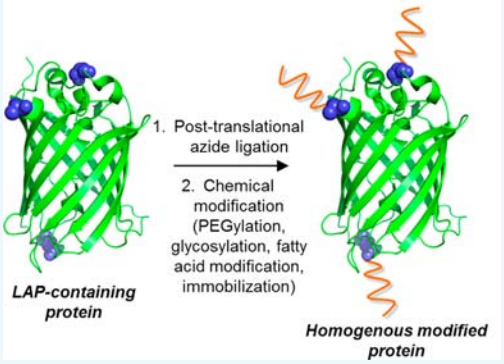
ABSTRACT: Approaches that allow bioorthogonal and, in turn, site-specific chemical modification of proteins present considerable opportunities for modulating protein activity and stability. However, the development of such approaches that enable site-selective modification of proteins at multiple positions, including internal sites within a protein, has remained elusive. To overcome this void, we have developed an enzymatic approach for multisite clickable modification based on the incorporation of azide moieties in proteins using lipoic acid ligase (LplA). The ligation of azide moieties to the model protein, green fluorescent protein (GFP), at the N-terminus and two internal sites using lipoic acid ligase was shown to proceed efficiently with near-complete conversion. Modification of the ligated azide groups with poly(ethylene glycol) (PEG), α-D-mannopyranoside, and palmitic acid resulted in highly homogeneous populations of protein−polymer, protein−sugar, and protein−fatty acid conjugates. The homogeneity of the conjugates was confirmed by mass spectrometry (MALDI-TOF) and SDS-PAGE electrophoresis. In the case of PEG attachment, which involved the use of strain-promoted azide−alkyne click chemistry, the conjugation reaction resulted in highly homogeneous PEG-GFP conjugates in less than 30 min. As further demonstration of the utility of this approach, ligated GFP was also covalently immobilized on alkyne-terminated self-assembled monolayers. These results underscore the potential of this approach for, among other applications, site-specific multipoint protein PEGylation, glycosylation, fatty acid modification, and protein immobilization.



INTRODUCTION

Chemical modification has widespread utility in modulating the activity and stability of proteins as well as monitoring biological processes (i.e., in vivo protein trafficking, protein folding) and as such is an important tool in many fields.[1−4] For example, the modification of proteins with natural and synthetic polymers and sugars may significantly enhance the circulatory lifetime and biological properties of protein drugs.[5−7] The covalent conjugation of responsive polymers (i.e., poly(*N*-isopropylacrylamide), azobenzene) has also been used to create novel protein switches that turn protein and enzyme activity "on" and "off".[8,9] To date, in addition to various polymers and sugars, proteins have been modified with a broad spectrum of molecular species, including labeling reagents (i.e., fluorophores, radiolabels), affinity tags, lipids, and derivatizing agents for attachment to supports.[10−12] The use of such modifying agents has ultimately found application in, among other areas, pharmaceutical development, drug delivery, tissue engineering, enzyme immobilization, and protein-based polymer engineering.[12−15]

A major challenge in the modification or derivatization of proteins with various agents entails preserving the protein's three-dimensional state such that the protein remains active and stable. Conventional approaches for chemical modification entail the formation of linkages via reaction with functional groups (i.e., amines or thiols) randomly located on the protein surface.[12] The covalent modification of proteins via reaction of random residues inherently leads to large heterogeneity in the resulting conjugates. Because the site of modification cannot be controlled, the sites that are critical for protein function may be partially or completely blocked upon modification, impacting protein activity.[16] Additionally, the activity of the protein may be further altered by the modification of residues that are involved in protein dynamics (i.e., hinge motions) as well as by the disruption of protein structure. Although natural residues, including cysteines or lysines, may be introduced at specific sites for modification, nonspecific conjugation may still occur if such residues at other sites are not mutated or removed. Furthermore, while techniques to modify the termini of a protein exist, these methods may not have the same desired effect as modification of internal sites and may be of little utility in cases where the termini are critical for function.

As a means to create highly uniform and active modified protein conjugates, the use of bioorthogonal conjugation chemistries presents considerable opportunities.[17,18] Bioorthogonal conjugation chemistries may specifically be enabled through the incorporation of noncanonical amino acids with non-native reactive handles that are unique in nature.[19−21] However, such approaches are hampered by low expression yields and incorporation efficiencies of noncanonical amino

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acids, which are amplified for more than one noncanonical amino acid.[22]

Herein, we present a novel bioorthogonal approach for the site-selective chemical modification of proteins at multiple positions via the enzymatic attachment of click reactive groups. This approach, which is both general and facile, entails the use of the enzyme lipoic acid ligase (LplA) to attach an azide-containing molecule (10-azidodecanoic acid) to a short peptide tag, which is a substrate for the ligase (Figure 1). The peptide

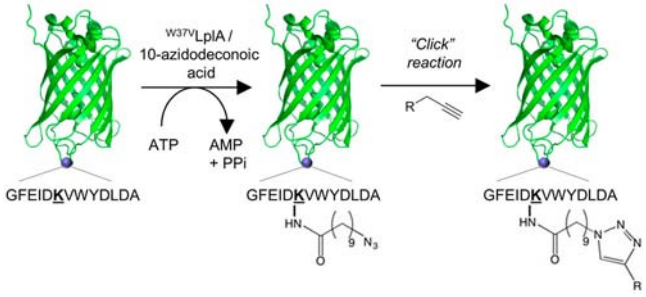

Figure 1. Schematic of the strategy for site-specific ligation of click reactive azide groups using lipoic acid ligase and subsequent chemical modification of GFP by click chemistry.

tag, which is a 13-amino-acid sequence known as the LAP sequence (GFEIDKVWYDLDA), may be inserted at the N- or C-terminus of the protein or in internal loop regions, provided its insertion does not perturb protein folding. Following the ligase-mediated reaction, the azide group may subsequently be modified via click chemistry with complementary alkyne-functionalized modifying agents or surfaces (i.e., for protein immobilization). The LAP/LplA system was initially developed by Ting and co-workers and used to label proteins with fluorophores in live cells as well as label isolated proteins in vitro.[23−27] Although previously demonstrated, the system has not yet been adopted for allowing general modifications to proteins at multiple positions for protein engineering. Similar enzyme-mediated approaches to conjugate proteins with modifying agents have been reported using biotin ligase, sortase, transglutamase, farnesyltransferase, phosphopantetheinyl transferase, and formylglycine generating enzyme.[28−30] While these approaches allow for single site modifications, such modifications are generally limited to the N- or C-terminus, nonspecific, and, in some cases, result in cleavage of the polypeptide backbone of the target protein.[31,32] Additionally, the substrates for these enzymes may not be readily available or easily synthesized, limiting widespread use. The practical use of these approaches may further be limited by slow kinetics of subsequent conjugation reactions.

The utility of the lipoic acid ligase-mediated approach for multi-site-selective chemical modification was demonstrated using the model protein green fluorescent protein (GFP). This approach was demonstrated through the design and modification of GFP constructs that contain the lipoic acid acceptor tag at single and multiple sites with poly(ethylene glycol) (PEG). Modification sites included the N-terminal position as well as multiple internal sites, showing the flexibility of this approach with respect to modification position. Ligated GFP constructs were also modified with sugar molecules as well as palmitic acid as a means of demonstrating site-specific glycosylation and modification with fatty acids. Finally, we also used this approach to site-specifically immobilize GFP on a self-assembled monolayer (SAM) with controlled orientation.

RESULTS AND DISCUSSION

Design and Expression of LAP-Containing GFP Constructs. Site-specific chemical modification of protein using the LAP/LplA system was investigated by designing GFP constructs containing the LAP sequence at terminal and internal positions. For demonstrating the utility of this approach, GFP is an ideal model protein due to its secondary structure, which contains a large number of flexible loop regions (Figure 2a).[33] These loop regions, which can accommodate the insertion of large polypeptides as well as tolerate altered connectivity,[34−36] provide numerous potential

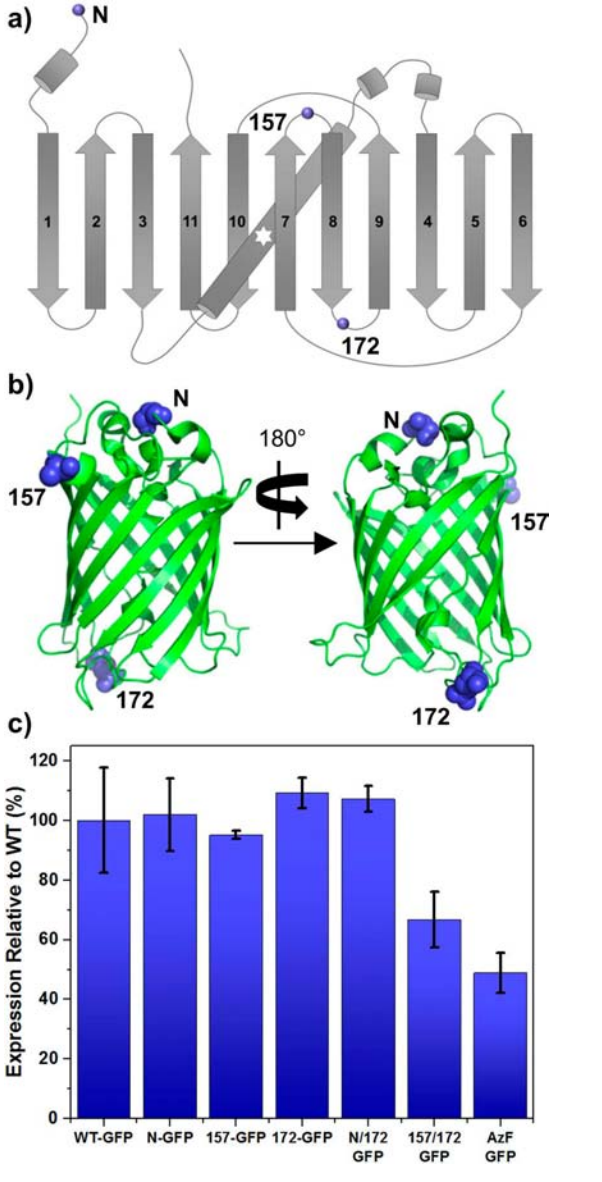

Figure 2. (a) Location of LAP insertion sites within GFP relative to primary sequence and secondary structure. Insertion sites are represented with blue spheres. (b) Position of LAP insertion sites in the context of folded GFP. Residues preceding insertion sites are depicted as spherical models in blue. (c) Expression data for all LAP-containing GFP constructs relative to WT-GFP. For AzF-GFP, the noncanonical amino acid AzF was incorporated at the N and 172 sites.

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LAP insertion sites on either end of the protein's β barrel structure. Furthermore, because the fluorescence of GFP is sensitive to its folding state and specifically the structure of its chromophore,[37] the structural impact of mutations and subsequent chemical modifications may easily be monitored. Accordingly, the fluorescence spectra of LAP-containing GFP constructs may be compared to that of wild-type GFP to determine if GFP folding and chromophore formation is perturbed. The intrinsic fluorescence of GFP can also be used to estimate expression levels of folded protein within cell lysate[38] and to observe surface immobilization, which can be imaged by fluorescence microscopy.

Five different LAP-containing GFP constructs were prepared by site-directed mutagenesis. Constructs with single LAP insertions at the N-terminus (N-GFP) as well as between Q157 and K158 (157-GFP) and between E172 and D173 (172-GFP) were designed. Additionally, constructs containing two simultaneous LAP insertions were prepared, the first with LAP sequences at the N-terminus and between E172 and D173 (N/172-GFP) and the second with LAP sequences between Q157 and K158 as well as between E172 and D173 (157/172-GFP). Notably, the sites at positions 157 and 172 are in solvent-exposed loops on either end of the β barrel structure (Figure 2b). As such, the 157/172-GFP construct contains LAP insertion sites at opposite ends of the protein, permitting modification of both ends of the protein simultaneously. Moreover, the site at position 172 is on the opposite side of the N- and C-termini and thus enables modification of a region of GFP that is not accessible by modifying the termini alone. To the best of our knowledge, although used to label sites within fusion constructs (i.e., between a protein and epitope tag such as polyhistidine tag),[26] the use of the LAP/LplA system for labeling sites that interrupt the primary sequence of a protein has not been reported previously. Although the commonly used sortase-tagging system has been used to label internal sites, cleavage by sortase requires the introduction of a disulfide bridge to prevent separation of the protein into fragments.[32]

All of the LAP-containing constructs, including double insertion constructs, were found to express well relative to WT-GFP as determined by measuring intrinsic GFP fluorescence (Figure 2c). To determine expression levels, the intrinsic fluorescence of GFP in crude *Escherichia coli* lysate for each construct was measured and normalized per liter of culture. The expression level of the double insertion construct 157/172-GFP was slightly lower than the other constructs, but still greater than that of GFP containing the azide-functionalized noncanonical amino acid 4-azido-L-phenylalanine. In this case, a double noncanonical amino acid construct (AzF-GFP) was expressed with 4-azido-L-phenylalanine at the N-terminus and at position 172 for comparison. The improved expression with the LAP sequence, which does not require altered transcriptional machinery, relative to AzF-GFP represents a major advantage of the LAP/LplA system. Additionally, use of the LAP tag evades translational inefficiencies of noncanonical amino acid incorporation such as release factor binding and misincorporation of native amino acids.

To confirm that fluorescence can be used to quantify expression in lysate, we confirmed that the fluorescence properties of the purified GFP constructs were similar. For all of the purified constructs, the excitation and emission maxima were 467 nm and between 509 and 511 nm, respectively. Similar fluorescence intensities, within 20% of WT-GFP, were also observed per micromole of purified protein (normalized through measurement of OD 280 nm) for each construct and used to calculate extinction coefficients. In addition to allowing the use of fluorescence measurements to compare expression levels, these results ultimately indicate that LAP insertion within GFP had little impact on protein structure, suggesting that the LAP sequence, in theory, may be inserted into flexible loop sites on other proteins as well without disrupting function.

Characterization of Ligase Reaction with GFP Constructs. Having expressed and purified LAP-containing GFP constructs, a critical next step was to investigate the efficiency and kinetics of the post-translational LplA-catalyzed ligation reaction. The GFP constructs (10 μM) were ligated with a 60-fold molar excess of the azide-containing substrate, 10-azidodecanoic acid (600 μM), using the previously described enzyme mutant, ^{W37V}LplA.[27] To determine the ligation efficiency as well as monitor the ligation rate, the addition of 10-azidodecanoic acid was monitored by electrospray ionization (ESI) mass spectrometry. As shown in the ESI spectra for N-GFP in Figure 3a, ligation of 10-azidodecanoic acid resulted in a shift of 195 Da, which is consistent with the expected mass increase. The peak for the expected ligation product increased over time while the peak for the native (i.e., unmodified protein) diminished until near-complete conversion was reached at approximately 3 h. Similar rates and extent of final conversion were observed for both single internal LAP-containing constructs relative to the N-terminal LAP construct (Figure 3b). No mass shift was observed either for the control ligation reaction with WT-GFP under the same conditions or

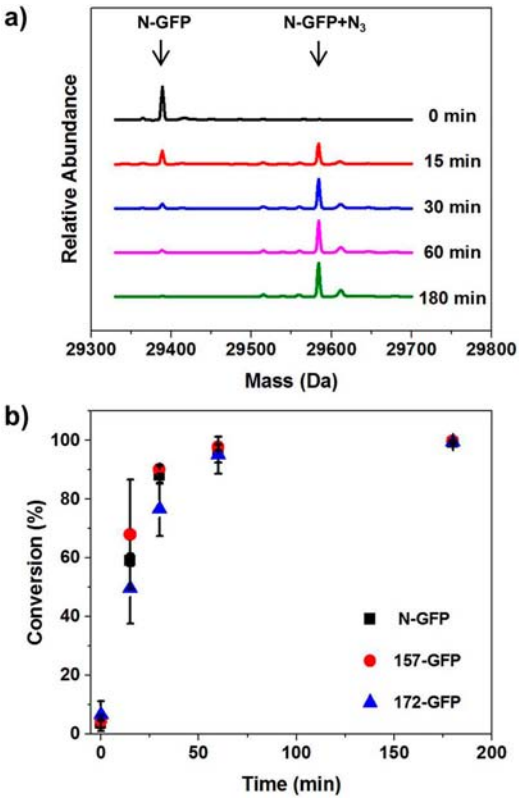

Figure 3. (a) ESI results for 10-azidodecanoic acid ligation to N-GFP. Relative abundance measurements are offset to illustrate the change in spectra as a function of reaction time. (b) Kinetics of ligation reaction for GFP constructs with single LAP insertions at positions *N*, 157, and 172.

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for LAP-containing constructs in reactions without ^{W37V}LplA, confirming that ligation is enzyme dependent and is specific to the LAP sequence.

In addition to the ligation reaction being highly efficient, ^{W37V}LplA is expressed well in *E. coli* and its substrate, 10-azidodecanoic acid, is easy to synthesize. This represents an advantage of the LAP/LplA system that is critical to its potentially widespread utility for protein modification. Another important advantage of the LAP/LplA system for modification is the ability to temporally control the introduction of azide groups at desired sites. Specifically, using this approach, an LAP-containing protein can be expressed and purified without azide functionalization, which can avoid reduction of the azide group. The reduction of azides when introduced via noncanonical amino acid incorporation is particularly problematic, as many proteins must be stored under reducing conditions for stability. Disruption of azides can also occur within the reducing environment in cells[39] or through UV-induced photolysis.[40] In all cases, azide reduction ultimately leads to greater heterogeneity within a protein population.

Apart from decreasing heterogeneity by limiting opportunities for reduction, temporal control of the LAP/LplA system also presents intriguing possibilities for modification strategies that simultaneously use noncanonical amino acid incorporation and LAP modification for sequentially labeling different sites. For example, a protein containing 4-azido-L-phenylalanine and an LAP site could potentially be modified by reaction of the noncanonical amino acid prior to ligation of the LAP site with an azide group. The combination of the LAP/LplA system with noncanonical amino acid incorporation could also be, in theory, although not demonstrated here, exploited to introduce more than one bioorthogonal functionality within a single protein. While the use of the LAP approach in conjunction with noncanonical amino acids for protein modification would presumably be compatible, additional studies are required to confirm this.

PEGylation of Ligated GFP Constructs. An interesting area in which the LAP/LplA system may be particularly useful is PEGylation of therapeutic proteins. The conjugation of PEG is a widely used strategy for improving the efficacy of therapeutic proteins by increasing circulatory lifetime in vivo.[5] Such an increase in circulatory lifetime can result from reduced proteolytic degradation, immunogenicity to the therapeutic protein, and renal clearance. However, nonspecific approaches to PEGylation lead to highly heterogeneous populations with individual protein molecules varying in the number and location of PEG attachment sites. Such heterogeneity requires often challenging purification of the desired PEG–protein conjugate, which may be necessary and critical to ensure safety and therapeutic efficacy.

To demonstrate the utility of the LAP/LplA system for site-specific protein PEGylation, the ligated GFP constructs were reacted with dibenzocyclooctyne–polyethylene glycol (DBCO-PEG; M_w 5 kDa). The reaction of DBCO-PEG with the ligated azide groups occurs via strain-promoted azide–alkyne cycloaddition and yields a stable covalent triazole linkage between the PEG and protein. Reaction products were characterized by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (Figure 4a) as well as SDS-PAGE (Figure 4b). In the case of SDS-PAGE, the presence of PEG in a particular protein band was confirmed by staining with iodine in addition to Coomassie staining. The reaction of ligated, LAP-containing constructs with DBCO-PEG resulted in large

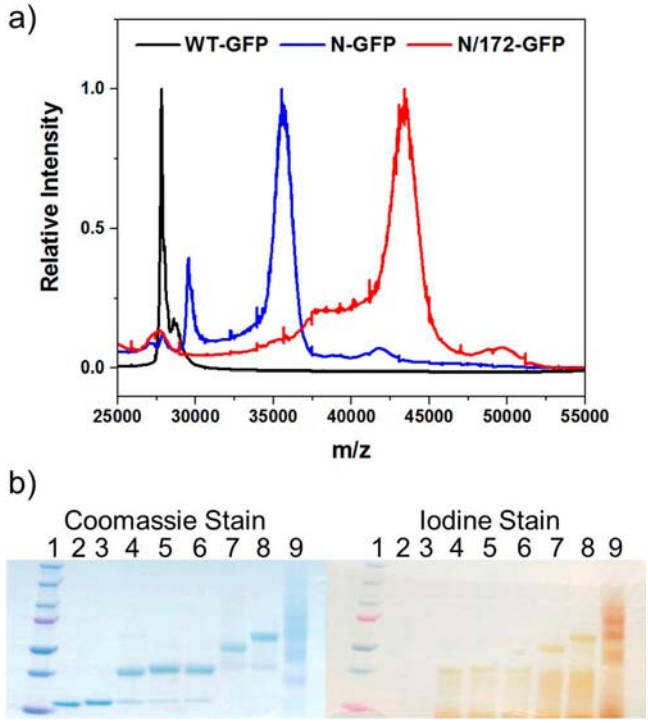

Figure 4. (a) Representative MALDI-TOF spectra of WT-GFP, ligated N-GFP+DBCO-PEG (labeled as N-GFP), and ligated N/172-GFP+DBCO-PEG (labeled as N/172-GFP). (b) SDS-PAGE of PEGylated GFP samples after 30 min reaction time. Coomassie staining indicates the presence of protein while iodine staining indicates the presence of PEG. Lane 1: Precision Plus Dual Color Ladder. Lane 2: WT-GFP. Lane 3: N-GFP. Lane 4: ligated N-GFP +DBCO-PEG. Lane 5: ligated 157-GFP+DBCO-PEG. Lane 6: ligated 172-GFP+DBCO-PEG. Lane 7: ligated N/172-GFP+DBCO-PEG. Lane 8: ligated 157/172-GFP+DBCO-PEG. Lane 9: WT-GFP+NHS-PEG.

proportions of protein migrating as higher molecular weight bands that stained with iodine, suggesting PEG attachment. MALDI data confirmed that these bands corresponded to PEGylated protein as the predominant peaks within the spectra matched expected molecular weights for the addition of one PEG molecule to single LAP-containing constructs and the addition of two PEG molecules to double LAP-containing constructs. Of note, when characterized by SDS-PAGE, the band for the PEGylated 157/172-GFP construct was unexpectedly higher than that for the PEGylated N/172-GFP construct. This apparent mass difference, which was not observed by MALDI (data not shown), was determined to be an artifact related to differences in SDS-PAGE mobility upon PEGylation at different sites.

For all of the GFP constructs, over 70% of the total protein was converted to the desired product within 30 min (as determined by densitometry of the gel images) with little residual unmodified protein. Moreover, no conjugation of DBCO-PEG was observed for control reactions with GFP constructs that were subject to ligation in the absence of 10-azidodecanoic acid, indicating that PEGylation was restricted to ligated LAP sites (data not shown). This combination of high site specificity and conversion enables the production of highly homogeneous PEGylated protein populations while reducing or eliminating the need to separate undesirable conjugates and

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potentially reducing the number of conjugates that require characterization.

To illustrate the advantage of the present approach for PEGylation over nonspecific techniques, WT-GFP was randomly reacted with methoxypolyethylene glycol propionic acid N-succinimidyl ester (NHS-PEG; 5 kDa M_w). Primary amines, such as a protein's N-terminus and the amine group of lysine residues, serve as reaction sites for NHS chemistry, and as such, there are 22 potential reaction sites in WT-GFP. Unlike with the LAP-mediated approach, conventional PEGylation with this chemistry yielded a heterogeneous population of PEG−protein conjugates with four major products as observed by SDS-PAGE (Figure 4b). These products correspond to proteins that vary in either the number or the location of attached PEG molecules, and thus the potential heterogeneity of this molecular population is substantial.

Although the limitations of nonspecific reaction chemistries for therapeutic protein PEGylation may alternatively be overcome with noncanonical amino acids,[38] one possible downside to this approach is that the incorporation of noncanonical amino acids may elicit unwanted immunogenicity. For example, *p*-nitrophenylalanine has been shown to trigger an immune response to otherwise self-tolerant proteins, including TNF-α and RBP4, when administered in mice.[41] Restricting residues to native amino acids may reduce the risk of such adverse responses, although additional studies would be required to determine if the insertion of the LAP sequence as well as ligation of 10-azidodecanoic acid is immunogenic, which may be highly protein dependent. Notably, the formation of a triazole linkage upon reaction of the azide group of 10-azidodecanoic acid may also contribute to the immunogenicity of the modified protein.

Glycosylation and Fatty Acid Modification of LAP-Containing GFP Constructs. In addition to PEGylation, site-specific glycosylation and fatty acid modification can modulate protein structure and function. However, site-specific glycosylation, in particular, represents a major challenge since many bacterial expression strains (e.g., *E. coli*) lack the necessary glycosylation machinery and pathways.[42,43] Additionally, eukaryotic expression generally results in diversity in position, length, and branching pattern of attached sugars, depending on organism and growth conditions.[44] Because many glycosylated proteins possess therapeutic potential, the development of approaches to produce well-defined glycosylated proteins has received considerable attention. Fatty acid modifications, though less prevalent in nature than glycosylation, can enhance transport across lipid barriers (e.g., the blood brain barrier) and stability in vivo (e.g., via binding albumin), and thus well characterized attachment of fatty acids also has important implications in improving the properties of therapeutic proteins.[11]

To further illustrate the breadth of feasible modifications, the LAP/LplA system was used to site-specifically glycosylate as well as modify GFP with fatty acids. This was demonstrated by attaching alkyne-functionalized α-D-mannopyranoside and palmitic acid to both ligated LAP sites on the 157/172-GFP construct using copper-catalyzed click chemistry (Figure 5). Unmodified 157/172-GFP has a molecular weight of 30 967 Da and increases in mass by 824 Da (theoretical) with ligation and subsequent mannose attachment at both sites and by 892 Da (theoretical) with ligation and palmitic acid attachment at both sites. The apparent shifts in mass when measured by MALDI-TOF were within error and thus consistent with these theoretical mass increases (see apparent masses in MALDI spectra in Figure 5). Protein glycosylation at internal positions has been demonstrated previously by Bertozzi and co-workers[45,46] using formylglycine generating enzyme, which converts a cysteine residue in the CXPXR recognition sequence to a formylglycine residue that contains a reactive aldehyde group. The LAP/LplA system may serve as an alternative to this approach for proteins in which insertion of the CXPXR tag promotes aggregation via intermolecular disulfide bond formation.

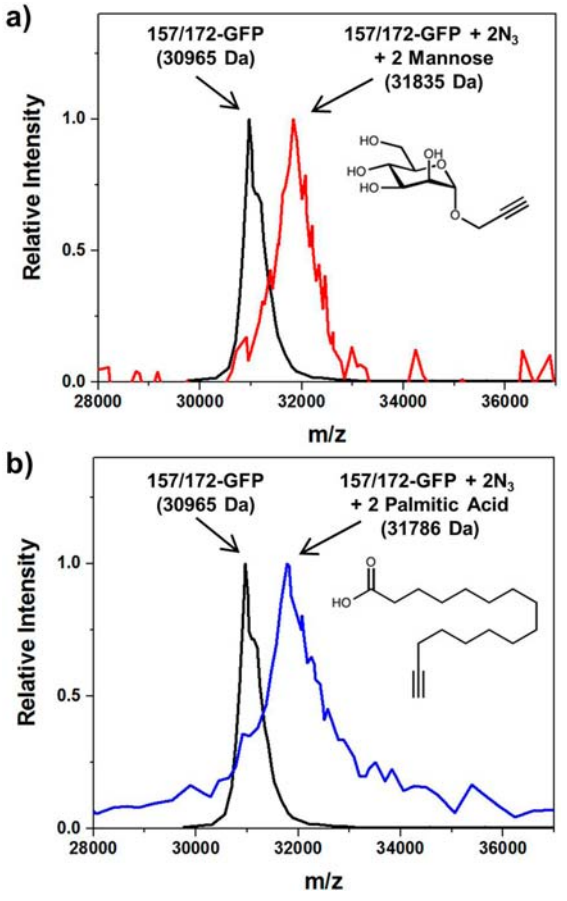

Figure 5. MALDI mass spectra for single charge states of 157/172-GFP with mannose (a) and palmitic acid (b) attachment at both internal LAP sites. The peak for 157/172-GFP is in black while the peaks for the attachment of two α-D-mannopyranoside and two palmitic acid molecules are in red and blue, respectively. Structures of mannose and palmitic acid are shown in each panel along with the apparent mass of each peak in the MALDI spectra.

Immobilization of Ligated GFP Constructs on Self-Assembled Monolayers. The bioorthogonal nature of the LAP/LplA system combined with the ability to design internal insertion sites suggests that it may also serve as a convenient strategy for orientation-controlled protein immobilization. To explore this possibility, ligated 172-GFP was immobilized on a DBCO-modified epoxide-terminated SAM, which was used as a model surface. Protein samples were thoroughly dialyzed to remove residual (i.e., free) 10-azidodecanoic acid prior to reaction of GFP with the surface. After extensive washing to remove noncovalently bound GFP, surfaces were imaged using fluorescence microscopy, illuminating the protein molecules bound on the surfaces (Figure 6a). The spot in the middle of

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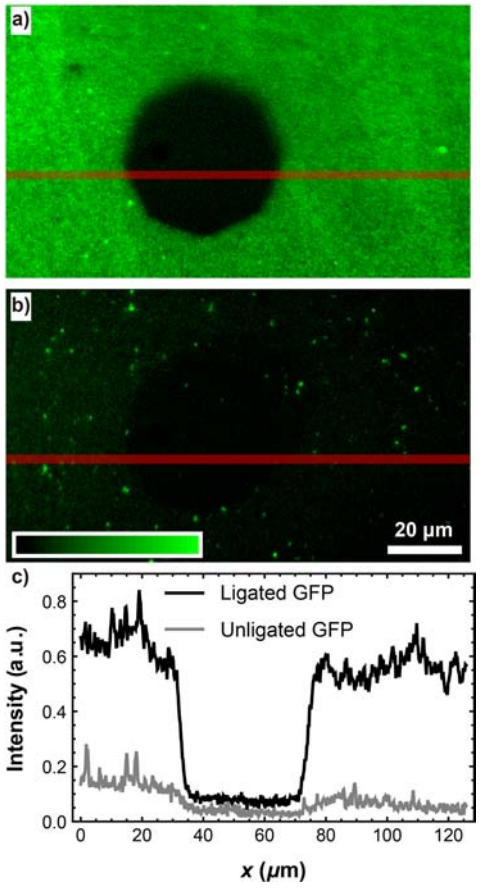

Figure 6. Immobilization of 172-GFP. (a) Image of surface after reaction of ligated 172-GFP with strained alkyne functionalized SAM. (b) Image of surface treated with WT-GFP control. The dark octagons in (a) and, less visible, in (b) were produced by photobleaching. The fluorescence intensity measured along the red lines in (a) and (b) was plotted in (c).

the fluorescent image was photobleached using long exposure times as a control to show the contrast between the fluorescence of the rest of the surface containing GFP and the spot. This contrast was large on the surface treated with ligated 172-GFP and negligible with WT-GFP (i.e., unligated GFP), which was used as a control, indicating a much higher presence of bound 172-GFP. To quantify this contrast, the relative fluorescence intensity across the red line in the fluorescent images of the surfaces was plotted using image analysis (Figure 6c). These results suggest that 172-GFP is immobilized, and that immobilization requires the azide functionality. This requirement implies that bound GFP molecules possess a single uniform orientation, and because the 172 LAP insertion site is on the opposite side of GFP's β barrel than both the N and C-termini, it is a particular orientation that would not be possible without an internal insertion of the LAP sequence. These results demonstrate the utility of the LAP/LplA system for controlling the orientation of immobilized biomolecules and thus its possible applicability to many areas of general importance, including biosensing and the creation of immobilized biocatalysts.

■ CONCLUSION

In summary, we have developed an enzymatic approach for multisite clickable modification based on the incorporation of azide moieties in protein using LplA. This approach was applied to the site-specific attachment of PEG, α-D-mannopyranoside, and palmitic acid to GFP as well as the immobilization of GFP on model SAM surfaces. Importantly, strong expression levels were obtained for the various GFP-tagged constructs, which contained single as well as double, simultaneous LAP insertions. Furthermore, the ligase-mediated modification of GFP with 10-azidodecanoic acid at the N-terminus and two internal positions was rapid and highly efficient. Our findings ultimately demonstrate the considerable potential utility of the LAP/LplA system for protein modification. Of particular advantage using this approach is the flexibility to incorporate multiple sites, including internal sites, at the same time. Additionally, this approach provides unprecedented temporal control of both azide functionalization as well as subsequent modification. Although we have demonstrated the breadth of feasible modifications for which this approach may be used, an interesting question that remains is the extent to which this approach may be used for the modification of proteins other than GFP. Specifically, future studies are necessary to address how well other diverse proteins and sites can accommodate LAP insertions and whether the LAP sequence can be engineered to be compatible with sites that are less accommodating to alteration than sites in flexible loop regions.

■ EXPERIMENTAL SECTION

Materials. The lipoic acid ligase (^{W37V}LplA) containing plasmid, pYFJ16-LplA(W37V),[27] was obtained from addgene (addgene plasmid 34838). The superfold GFP construct, pET-stGFP,[47] was kindly provided by David Liu of Harvard University and the tRNA/synthetase pair for 4-azido-L-phenylalanine incorporation was provided in the form of the pDule2 pCNF RS plasmid[48] courtesy of Ryan Mehl of Oregon State University. Mutagenic primers were purchased from Integrated DNA Technologies (Coralville, IA). DBCO-PEG (M_w 5 kDa) was purchased from Jena Bioscience GmbH (Jena, Germany), propargyl α-D-mannopyranoside from LC Scientific Inc. (Concord, Ontario) and 15-hexadecynoic acid (palmitic acid alkyne) from Cayman Chemical (Ann Arbor, MI). The ligand tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) for copper-catalyzed click reactions was obtained from Click Chemistry Tools (Scottsdale, AZ), 4-azido-L-phenylalanine from Chem-Impex International, Inc. (Wood Dale, IL), and 5,6-epoxyhexyltriethoxysilane from Gelest (Morrisville, PA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cloning, Expression, and Purification of LAP-Containing GFP Constructs. The LAP sequence GFEIDKVWYDL-DA was introduced into stGFP within pET-stGFP using the QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Primers for mutagenesis were designed with 25 to 30 bp of DNA complementary to pET-stGFP flanking both sides of the LAP sequence. Successful mutagenesis for all LAP-containing stGFP constructs was confirmed via sequencing (Eurofins Genomics, Huntsville, AL).

Wild type GFP and LAP-containing constructs were expressed in BL21 DE3 *E. coli* cells using ampicillin as a selective marker. After initial inoculation, transformed cells were grown in LB to an OD of ~0.6 at 37 °C with shaking at 200 rpm and subsequently induced with 1 mM IPTG. Cells were then harvested after an overnight incubation at 25 °C and at 200 rpm via centrifugation and lysed by homogenization in 50 mM Tris (pH 8.0), 250 mM NaCl, 5 mM imidazole, 0.01%

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β-mercaptoethanol, and 2% glycerol. The resulting lysate was clarified by centrifugation and loaded onto Ni^{2+} charged Bio-Scale Mini immobilized metal affinity chromatography cartridges (Bio-Rad, Hercules, CA). Protein containing an N-terminal polyhistidine tag was eluted in lysis buffer containing 150 mM imidazole and subsequently dialyzed into 20 mM sodium phosphate buffer (pH 7.0). Purified protein was flash frozen in liquid nitrogen and stored at −80 °C.

Constructs for 4-azido-L-phenylalanine incorporation were similarly prepared via mutagenesis except that the amber stop codon TAG was inserted in place of the LAP sequence. The resulting constructs were co-transformed with pDule2 pCNF RS in BL21 DE3 cells and expressed using the same procedure as the other GFP constructs with the exception of the addition of 2 mM 4-azido-L-phenylalanine and the second selective marker, spectinomycin, to the induction media.

GFP Fluorescence Measurements. Excitation spectra (300−550 nm) were obtained for the purified GFP constructs using a Fluoromax-4 spectrofluorometer (Horiba Scientific, Edison, NJ). The excitation maxima for each was found to be 467 nm, which was subsequently used to excite each construct to obtain emission spectra (450−600 nm). Relative stGFP expression levels were obtained by measuring the fluorescence of clarified cell lysate from 250 mL induction volumes. The fluorescent intensities of these samples were converted to protein concentrations with extinction coefficients obtained from purified variants. Total protein expression of each variant was normalized to WT expression levels. Expression data was obtained in duplicate.

Synthesis of 10-Azidododecanoic Acid. The substrate for lipoic acid ligase, 10-azidodecanoic acid, was synthesized following the method of Yao and co-workers.[27] Briefly, 1 g (3.98 mmol) of bromodecanoic acid was added to 10 mL of *N,N*-dimethylformamide (DMF). To this, 0.5 g (7.69 mmol) of sodium azide was added and allowed to stir at room temperature overnight. The reaction was monitored by thin layer chromotagraphy using 1:2 hexanes/ethyl acetate after which the solvent was removed under vacuum. Following solvent removal, 15 mL of 1 M HCl was added to the resulting product, which was then washed 3 times with 15 mL of ethyl acetate. The organic layers were combined and dried over sodium sulfate and subsequently placed under vacuum. The product was then separated by silica gel chromatography using a solvent gradient of hexane and ethyl acetate. The identity of the acid was confirmed by infrared spectroscopy and nuclear magnetic resonance. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.23 ppm (t, 2H), 2.32 ppm (t, 2H), 1.57 ppm (m, 5H), 1.31 ppm (m, 9H). IR (15%EtOAc/Hexanes): 1706, 2093 cm^{-1}.

Ligation Reaction. Ligation of 10-azidodecanoic acid to LAP-containing GFP constructs was performed as described by Yao and co-workers.[27] For this, LplA was expressed in BL21 DE3 cells from the pYFJ16-LplA(W37V) plasmid. Purified LplA was dialyzed into 20 mM Tris (pH 7.5), 0.01% β-mercaptoethanol, and 10% glycerol, flash frozen in liquid nitrogen, and stored at −80 °C. Ligation reactions containing 0.1 μM LplA, 10 μM GFP, 600 μM 10-azidodecanoic acid, 2 mM ATP, 2 mM $MgCl_2$, and 25 mM phosphate buffer (pH 7.2) were incubated at 30 °C. Reactions were quenched at various time points via chelation by the addition of EDTA to a final concentration of 300 mM. Quenched ligation products were characterized via ESI mass spectrometry (Synapt G2), which was preceded by exchanging the buffer with 40% acetonitrile and 0.1% formic acid. Spectra were deconvoluted with *MaxEnt*, and conversions were obtained by dividing the peak height of ligated GFP by the sum of ligated and unligated GFP peak heights. Ligated protein was thoroughly dialyzed to separate the ligated protein from residual components of the ligation reaction before being used in subsequent click reactions. Protein samples were dialyzed in seamless cellulose dialysis tubing from Fisher Scientific (Waltham, MA) with a molecular weight cutoff of 12−16 kDa for 12 h in 4 L of dialysis buffer. The dialysis buffer was changed frequently (a minimum of three times) to facilitate dialysis.

PEGylation of GFP Constructs. Nonspecific PEGylation of wild type GFP was performed in 50 mM sodium phosphate buffer (pH 7.5) and at room temperature for 3 h via reaction with NHS-PEG. A 5:1 molar ratio of PEG-to-primary amine was used for the reaction. Site-specific PEG attachment using DBCO-PEG was similarly performed in 50 mM sodium phosphate buffer (pH 7.0) using a 25 molar excess of DBCO-PEG for each ligation site to obtain samples for MALDI and a 50 molar excess of DBCO-PEG to obtain samples for SDS-PAGE. The click reaction was incubated at room temperature for 0.5−6 h. Reaction samples were removed at varying time points and quenched with 100 mM tris(2-carboxyethyl)phosphine. Following the reaction, the reaction products were characterized by SDS-PAGE and MALDI-TOF mass spectrometry. For iodine stained SDS-PAGE gels, the gels were stained for 10−30 min and imaged after destaining with water. The iodine stain consisted of 1.3% (w/v) iodine, 1.0% (w/v) potassium iodide, and 2.5% (w/v) barium chloride in a 0.6 M HCl solution. After imaging, gels were restained with Coomassie and reimaged. Images of Coomassie stained gels were analyzed quantitatively by densitometry using *ImageJ*.[49]

Copper-Catalyzed Click Modification of Ligated GFP Constructs. Propargyl α-D-mannopyranoside conjugation to ligated GFP by copper-catalyzed azide−alkyne cycloaddition was performed using the optimized conditions described by Hong et al.[50] Initially, 865 μL of GFP (10 μM in 50 mM sodium phosphate, pH 7.0) was added to 20 μL of propargyl α-D-mannopyranoside (20 mM in deionized water) in a microcentrifuge tube. To the GFP mixture, 15 μL of a solution of $CuSO_4$ and THPTA, which was prepared by initially mixing 50 μL of 20 mM $CuSO_4{\cdot}5H_2O$ (in deionized water) and 100 μL of 50 mM THPTA (in deionized water) for 20 min, and 50 μL of aminoguanidine hydrochloride (100 mM in deionized water) was added. The click reaction was initiated by the addition of 50 μL of sodium ascorbate (100 mM in deionized water), which was allowed to proceed for 4 h at 37 °C.

Palmitic acid alkyne conjugation to GFP was performed using conditions identical for the α-D-mannopyranoside attachment except the palmitic acid-alkyne stock was prepared in DMSO. Additionally, the click reaction was performed in the presence of 2% sodium deoxycholate to solubilize the palmitic acid.

Following the attachment of the α-D-mannopyranoside or palmitic acid, the resulting reaction mixtures were dialyzed with deionized water. The GFP conjugates were then analyzed by MALDI-TOF.

Click Immobilization of Ligated GFP Constructs. Glass cover slides were initially washed with detergent and thoroughly rinsed with ultrapure water. The slides were subsequently immersed in warm piranha solution for 1 h followed by thorough rinsing with purified water, drying with ultrapure nitrogen, and exposure to UV-ozone for 15 min. The surface was then functionalized with epoxide groups by forming

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Bioconjugate Chem. 2015, 26, 1104−1112

a SAM of 5,6-epoxyhexyltriethoxysilane. The SAM was formed by exposing the cleaned glass to vapors of a mixture of the silane (10% v/v), *n*-butylamine (5% v/v), and toluene (85% v/v) for 20 h. Attachment of the silane was confirmed by using a custom-built goniometer to measure the static water contact angle. The static water contact angle after silane modification was 36.3° ± 0.8° where the error represents the standard deviation from 12 total measurements over 4 slides.

Following deposition of the SAM, the terminal epoxide groups were reacted with dibenzocyclooctyne-amine (DBCO-NH_2) to introduce alkyne groups to the surface. For this reaction, DBCO-NH_2 was dissolved in DMSO at a concentration of 5 mg/mL and added to a borate buffer (100 mM, pH 9.5), resulting in a final concentration of 1 mM. The DBCO-NH_2 solution was subsequently added to a small Petri dish containing the epoxide-modified surfaces to start the coupling reaction. The reaction mixture was placed in an orbital shaker at 37 °C and allowed to react for 30 h. After the reaction, the surfaces were thoroughly washed with deionized water and then immersed 1% w/v ammonium chloride (in 100 mM borate buffer, pH 9.5) overnight at 37 °C to quench the remaining epoxides. To attach GFP, 10 nM of the ligated GFP (or WT-GFP for control) in sodium phosphate buffer (100 mM, pH 7.0) was allowed to react with the DBCO functionalized surface for 4 h under gentle agitation. Finally, noncovalently bound GFP was washed from the surface by gently shaking with sodium phosphate buffer (100 mM, pH 7.0) for 4 h at 37 °C. The rinse buffer was replaced approximately every 30 min during washing.

Imaging of GFP Attachment Using Epifluorescence Microscopy. Glass slides were imaged in epifluorescence mode using a Nikon TE-2000 microscope, 60× objective, and an Andor iXon 3 EMCCD camera (model DU 888). Light was provided by a metal halide arc lamp (X-Cite 120, Lumen Dynamics) and a filter cube to select light from 450 to 490 nm for excitation and capture light from 505 to 550 nm for fluorescence imaging. A spot on the glass slide was photobleached for 10 min at a power density of 20 W/cm^2 using an iris to block light from the surrounding area. Following bleaching, a video was recorded at ∼19 fps (52 ms per frame) while the iris was opened. The 10 frames immediately after the iris was fully open were averaged to image the contrast between bleached and unbleached areas of the surface. All surfaces were imaged using identical camera settings and illumination power density.

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Notes

The authors declare no competing financial interest.

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